LESSON 35:
PLANT TISSUE CULTURE

Learning Objectives

In this lecture, you will learn

- Methodology involved in plant tissue culture techniques
- Benefits of plant tissue culture techniques
- Maintenance of plant cell lines
- Equipments and media for the development of plant cell lines

Plant cell and tissue culture

Introduction

Definition

Plant tissue culture (PTC) is a generic description which includes plant protoplast, plant cell, plant tissue, plant organ and plant culture, where these various types of culture involve, as a common factor, the growth of microbe-free plant material in an aseptic (sterile) environment, such as sterilized nutrient medium in a test tube.

Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, etc.), disease, political and labour instabilities in the producing countries (often Third World countries), uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Thus, the production of useful and valuable secondary metabolites in large bioreactors located in the consuming country is an attractive proposal. Additional advantages of such processes include: controlled production according to demand and a reduced and requirement.

Plant Tissue Culturing Techniques have become especially important in the agricultural community over the past ten years. During this time period, plant tissue culture has effectively moved from the confines of small laboratories and has taken its place among some of the more mainstream, broad-scale techniques employed by the agriculture industry.

Tissue Culture Centers

However, this technology is still being developed and despite the advantages outlined above, there are a variety of problems to be overcome before it can be adopted on a wide scale for the production of useful plant secondary metabolites. The success of Mitsui Petrochemical Industry Co. Ltd. in Japan in producing shikonin on a commercial scale from *Lithospermum erythrorhizon* cultivations and that of Nitto Denko Co. Ltd. also in Japan in mass production of *Panax ginseng* or ginseng cells using 20 kL tanks have demonstrated that many of the problems can be overcome with perseverance. The economic feasibility of these processes is another question and this will be dealt with in a separate section.

Plant Tissue Culture, more technically known as micropropagation, can be broadly defined as a collection of methods used to grow large numbers of plant cells, in vitro, in an aseptic and closely controlled environment. This technique is effective because almost all plant cells are totipotent – each cell possesses the genetic information and cellular machinery necessary to generate an entire organism. Micropropagation, therefore, can be used to produce a large number of plants that are genetically identical to a parent plant, as well as to one another.

Figure 5-10 - Areas of Tissue Culture Collection

The Standard Protocol for Plant Tissue Culture

The standard protocol for performing plant tissue culture experiments is fairly basic. First, it is essential that a sterile environment be created. The medium used to grow the plant tissue, the plant tissues themselves, and the environment

surrounding the tissue culture, must be free of all possible contaminants. The presence of any bacterial, fungal, algal, or viral contaminants could potentially rob the desired plants of the nutrients provided by the culture medium and have devastating effects upon their growth (Kyle, 2002). Once a sterile environment has been established, tissue can be collected from the plant's leaf, shoot, bud, stem, or root (see figure 5-10). Because each of these cells is totipotent, each has the potential to express an entire organism (see figure 5-11). The tissue sample can then be placed on an aseptic (free of microorganisms), nutrient-rich medium where its cells will begin to grow and develop into the desired plant product . The nature of the medium and the nutrients that it contains is dependent upon the type of plant being grown and the properties that the grower wishes to express. Finally, the developing tissue should be maintained in a closely controlled chemical and physical environment, such as a greenhouse, to achieve the best results.

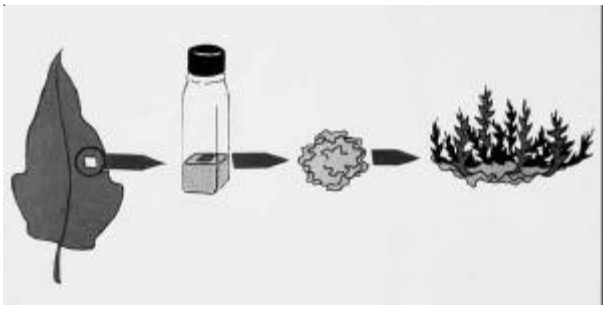

Figure 5-11 - The Basic Steps of Micropropagation

Basic Principle in Plant Tissue Culture Technique

Plant tissue culture or micropropagation is the aseptic culture of cells, pieces of tissue, or organs. It is possible to regenerate new plants from small pieces of plant tissue because each cell of a given plant has the same genetic makeup and is totipotent, that is, capable of developing along a "programmed" pathway leading to the formation of an entire plant that is identical to the plant from which it was derived. Using micropropagation, millions of new plants can be derived from a single plant. This rapid multiplication allows breeders and growers to introduce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. Micropropagation also can be used to establish and maintain virus-free plant stock. This is done by culturing the plant's apical meristem, which typically is not virus-infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants.

Micropropagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success. The process of micropropagation can be divided into four stages:

- Initiation stage. A piece of plant tissue (called an explant) is cut from the plant, disinfested (removal of surface contaminants), and placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.
- Multiplication stage. A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells.
- Rooting or preplant stage. Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. Auxins are plant growth regulators that promote root formation. For easily rooted plants, an auxin is usually not necessary and many commercial labs will skip this step.
- Acclimatization. A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

Organ Cultures

Organ cultures involve an organ, such as a bud, and manipulation of cultural conditions so that this organ remains organized throughout all of the cultural procedures. Callus cultures and suspension cultures, in contrast, begin with pieces of tissues and cells from an organized part of the plant but cultural conditions are manipulated to create an unorganized state, e.g. a callus, single cells or aggregates of cells. An organ consists of tissue which, in turn, consists of cells; it is a cooperative aggregation of tissues and cells performing a particular function. By maintaining the organization which characterizes an organ, we maintain its integrity and function in organ culture in the same way as it is maintained in natural methods of vegetative propagation, e.g. runners, and man-induced methods of clonal propagation, e.g. budding. This being so we would not expect any more nor any less variation in our clones by organ TC-propagation than by other methods of vegetative propagation. Organ TC-propagation is comparable to the use of a cutting but on a miniature scale; in contrast to a cutting, it has the potential to develop a very large number of clonal individuals. However, one snag is that if the bud used to start an organ culture turns out to have been a "sport", then we would finish up with a large population of this sport. The cultural conditions themselves should not induce "sporting"; if they did, then the factor in the cultural conditions responsible for this induction would have to be determined and corrected.

Initiation of Organ Cultures

When we begin work with a cultivar, we first have to get sufficient of its buds into aseptic culture so that we can test out a range of cultural conditions so as to discover ways of inducing one or other type of multiplication. Because it is often difficult and time consuming to obtain sufficient aseptic bud cultures from mature plants, particularly those growing in the open, we often test out a range of cultural conditions with aseptic seedlings. Seeds are, in general, easier to disinfest and although information on the responses of nodes from seedlings may not be directly applicable to nodes from adult material, it nevertheless is usually a fair indication of the approximate requirements for the culture of nodes and buds from adult material. Thus this procedure of experimenting

with easily obtainable aseptic seedlings to screen numerous cultural combinations of media and incubation, usually means that we need test a narrower range of combinations with the scarcer, more difficult to obtain, aseptic adult material. In practice, we would simultaneously experiment with seedling material and strive to get adult material into aseptic culture on a simple medium. Usually, by the time the screening experiments with seedling material have been completed, sufficient aseptic adult material would be available for testing on combinations selected from the seedling experiments.

Rapid Multiplication of Organ Cultures

Getting an organized structure such as a bud into aseptic culture is a time consuming process and, in general, the earliest stages of organ micropropagation involve loss of cultures through either microbial contamination or death of cultures. Multiplication rates also tend to be slow at first but later, if the cultural conditions are satisfactory, much higher rates of multiplication are achieved - the plant material's physiology seems to change and a rapid proliferation of buds can be obtained.

What type of multiplication do we try to attain? One type results in the development of the cultured bud into a shoot, like a branch, with normal or greater elongation of internodes, so that the new leaves and their axillary buds are well separated from each other. Under appropriate cultural conditions, the cultured bud may develop a shoot with 5 or more nodes over a 2 month period of incubation; each of these nodes can then be excised and cultured on fresh medium and, after a further 2 month period of incubation, each subcultured node would develop a shoot with 5 or more nodes. Thus, we would have a multiplication rate of x5 every 2 months, which would give us 5 x 5 x 5 x 5 x 5 x 5 (= 56 = 15,625) nodes for further culture or for establishment, after 12 months.

Another type of multiplication which has been attained involves the inhibition of apical dominance of the cultured bud. When we prune a shrub, we remove the growing points, i.e. apices, of the stem and branches, and thus remove their apical dominance (i.e. their inhibitory function) over the buds in the axils of leaves on the remaining parts of the stem and branches. These no longer inhibited buds develop into shoots and branches. In organ culture, we have been able with some species to overcome apical dominance by chemical means. Thus, under appropriate cultural conditions, the initial bud cultured grows only slightly before developing lots of axillary buds which are separated from each other by only very short internodes. Each of these axillary branches grows slightly and then develops its axillary buds, and so on. Often the resulting cultures bear little resemblance to the normal plant and take on a grass like or moss like habit. Later, these buds are planted on different media or are exposed to different incubation conditions to restore them to their normal habit. Care has to be exercised with this type of multiplication to ensure that a reversal of habit in culture is possible; cultures exposed to this type of multiplication system are also prone to initiate adventitious bud formation in addition to axillary buds. This type of multiplication system is capable of producing in excess of 10 buds during a 2 month incubation period, that is 10 x 10 x 10 x 10 x 10 x 10 (= 106 = 1,000,000) buds over a 12 month period.

The Induction of Root Formation in Organ Cultures

Up until now we have not discussed the procedures leading to the induction of root formation in organ cultures because we do not need roots when we are endeavouring to multiply the shoot system. A key factor in the multiplication of the shoot system appears to lie with the cytokinins; relatively high concentrations of cytokinins, in several species so far tested, favour rapid multiplication, and particular cytokinins favour one or other types of multiplication. The factors which are usually tested to induce root formation include either the omission of, or very low concentrations of, cytokinins in the culture medium, i.e. cytokinins appear to be inhibitory to root formation. Other factors include the incorporation of particular auxins, e.g. IBA, NAA, into cytokinin-free media, and the use of liquid media with filter paper support for the culture.

Planting out of Cultured Plants

There is a great need for more research into the planting out of cultured plants not so much to find out HOW to do it but rather to find out how to do it economically and in a manner suited to automation.

We are dealing with very small shoots or buds which are very "soft" because of their previous cultural environment, and which do not photosynthesize; their roots, if any, may also not be functional outside of the cultural environment.

Successful planting out depends as much on laboratory manipulations as on nursery procedures.

Adventitious Buds

It was earlier mentioned that cultures which are being rapidly multiplied by means of overcoming apical dominance are prone to initiate adventitious bud formation in addition to developing axillary buds. The induction of adventitious bud formation from leaves is already used in the normal propagation of *Peperomia* and *Begonia* , for example, and was recently demonstrated in tissue culture when cauliflower internodal explants were used. Indeed, a micropropagation method of inducing adventitious buds from internodes, petioles and leaf sections has the attraction that much more plant material, say of a rare sport or hybrid, would be available to start cultures. However, caution should be exercised with such systems for micropropagation until it is established whether such adventitious buds are clonal with the parent. The reason for this caution lies in the existence of polyploid cells and cells with other chromosomal differences within a plant. This is not a general rule inasmuch as some of the few plants studied have not shown evidence of these phenomena. Neither does the presence of such cells necessarily mean that they would participate in the formation of adventitious buds; in fact, polyploid cells, particularly those with high levels of ploidy, are often short lived, e.g. root cap cells, and they have been associated with cells showing high levels of metabolic activity.

Callus and Suspension Cultures

Polyploid cells and cells with various types of chromosomal variations from the normal for the species have often been reported for callus and suspension cultures, and thus plants

which are induced to form from such cultures, either by the induction of adventitious buds or by embryogenesis, may arise from cells of an abnormal type. Nevertheless, the presence of cells with other than normal genomes does not necessarily prevent the formation of adventitious buds and embryos with the normal chromosomal type of the species. On the other hand, this variation in genome could lead to the formation of interesting and valuable new strains. However, for clonal propagation, we have at present to use the callus and suspension culture methods with extreme caution. If and when we solve the problems associated with genome instability, suspension culture in particular would be of considerable importance.

The problems of callus and suspension culture do not rest with genome instability alone. Although it is relatively easy to make callus and suspension cultures of many species, including woody species, so far we have been able to induce organogenesis and/or embryogenesis with very few species. For example, with tobacco, it is easy to obtain aseptic material to start a callus culture by excising pieces of pith from the stem. Such pieces form callus, i.e. a mass of cells with none of the organization associated with the pith, when placed on an agar based culture medium containing both an auxin and a cytokinin at 10µM and 1µM respectively, for example. To initiate adventitious bud formation from these calluses, pieces are subcultured to the same medium except that it may contain 10µM cytokinin and no auxin.

Carrot calluses, prepared in a similar way to tobacco calluses, are placed in liquid medium and placed on a shaking machine orbiting at about 150 r.p.m. The liquid medium may contain about 5µM of the auxin 2,4-D but have no added cytokinin in it. The rotation breaks up the callus and, after 4 to 6 weeks, we would have a fine suspension of single cells and of groups of cells. When these are removed and spread thinly on the surface of an agar based medium which is hormone free, i.e. does not contain 2,4-D, many of the cells form embryos which can develop into whole plants. The same effect can be achieved by putting the cell suspension into 2,4-D free liquid medium and by continuing the rotation of cultures.

Materials and Methods

The Selection of a Medium and Nutritional Components of Tissue Culture Media

The choice of a particular medium depends mainly on the species of plants, the tissues or organ to be cultures, and the purpose of the experiment. Moreover, the choice and concentration of hormones are the most important consideration in preparation of the medium for monocotyledon and dicotyledon tissue cultures. A suitable starting point for the initiation of callus or dicotyledon tissue explant will be the preparation of the MS basal medium. One characteristic of this medium is its relatively high concentration of nitrate, potassium and ammonium ions in comparison with other nutrient media. For monocotyledon culture, it is suggested to start with the Schenk and Hilderlrandt medium. The success of the application of in vitro methods is due largely to a better understanding of the nutritional requirements of cultured cells and tissues. Since plant tissue cultures need more complex nutrient than the whole plants and they are seldom autotrophic, hence the optimum nutrition is required. In general, plant tissue culture medium contains 5 groups of ingredients: inorganic salts, carbon sources, vitamins, plant growth regulators and organic supplement.

Inorganic Salts

The inorganic salts consist of essential macroelements which are elements that plants need a large amount such as N, P, K, Ca, Mg, S and microelements which are elements that plant need a trace amount such as Fe, Mn, Zn, B, Cu, Mo and Co. To ensure the availabilitiy of iron over a wide range of pH, it is prepared separately in a chelated form as the sodium salt of ferric ethylenediamine tetra-acetate (Na-FeEDTA).

Carbon Sources

All culture media require the presence of a carbon as energy source since plants or explants are not completely autotrophic. The standard carbon source is saccharose (sucrose) or glucose which is usually added with the concentration of 2 to 3 %. Fructose also can be used but is generally much less suitable. Other carbohydrates including lactose, maltose, galactose and starch are poor carbon sources. The choice of sugar use depends mainly on the cultured plant tissue and the purpose of the experiment.

Vitamins

Vitamins have catalytic functions in enzyme systems and are requires only in trace amounts. Most media contain myo-inositol at a concentration of 100 mg/l which improves cell growth. Other vitamins that have been used in plant tissue culture media are (alternative names and concentrations used in mg/l in brackets): thiamin hydrochloride (vitamin B1; 0.1-5.0), nicotinic acid (niacin; 0.1-5.0), pyridoxine (vitamin B6; 0.1-1.0), Ca-panthothenate or panthothenic acid (0.5-2.5), folic acid (vitamin M; 0.1-0.5), riboflavin (vitamin B2; 0.1-10), ascorbic acid (vitamin C;1-100), biotin (vitamin H; 0.01-1.0), para-aminobenzoic acid (0.5-1.0), tocopherol (vitamin E; 1-50).

Plant Growth Regulators

Plant growth regulators which have different regulatory effects on growth and development in whole plants and plant tissue cultures are add into a nutrient medium. It is accepted that without regulators, in vitro culture is often impossible. Plant growth regulators which are often used in plant tissue culture are the following.

Auxins

The most frequently employed auxins are IAA, IBA, NAA and 2,4-D. IAA, a naturally occurring auxin is added in concentration of 0.01-10 mg/l. The synthetic and relatively more active auxins (IBA, NAA and 2,4-D) are used at the concentration of 0.001-10 mg/l. Other auxins which may be used in plant tissue culture include 2,4,5-T and 4-CPA. The most effective auxin of callus proliferation for most cultures is 2,4-D, but unfortunately it strongly suppresses organogenesis and should not be used in experiments involving root and shoot initiation . Auxins generally cause: cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots; the inhibition of adventitious axillary shoot formation, and often embryogenesis in suspension culture. With low auxin

concentration, adventitious root formation predominates, whereas with high auxin concentration, root formation fails to occur and callus formation takes place.

Cytokinins

Cytokinins are derivatives of adenine , which promote cell division, regulate growth and development in plant tissues. The most widely used cytokinins in culture media are kinetin, BA, 2iP, PBA and Zeatin. Kinetin, BA ,2iP and PBA are synthetic compounds, whereas zeatin occurs naturally. In evaluating the requirement for cytokinin by specific cell line, concentrations in the range of 0.01 to 10 mg/l and sometimes as high as 100 mg/l are tested. By varying the ratio of auxin and cytokinin in the medium, several investigators have succeeded in the regulation of cell division, cell elongation, cell differentiation and organ formation.

Other Regulators

Other types of hormones which may be used in plant tissue culture include gibberellins (GA3), ethylene, abscisic acid and oligosaccharins. GA3 induce elongation of internodes and the growth of meristems or buds in vitro. They may also break dormancy of isolated embryos or seeds. GA3 usually inhibit adventitious root formation as well as adventitious shoot formation. Ethylene is used in the initiation of bud. Abscisic acid has a negative influence on in vitro cultures, however, it probably promotes callus growth and embryogenesis incidentally. Oligosaccharins are chemical messengers with specific regulatory properties which were discovered recently. These compounds can regulate not only by triggering the plant's defense against pathogens and other types of stress, but also by regulating the rate of growth and differentiation into roots, flowers and vegetative buds.

Organic Compounds

The trend in plant tissue culture has been attempted to define the constituents of a given medium and eliminate the use of crude natural extracts. Such products as peptone, yeast extract, and malt extract are used infrequently today. However, the use of natural extracts should not be ignored when chemically defined mixture fails to produce the desired results. Coconut water, orange juice and tomato juice are important organic supplements. Coconut water is commonly used at 2 to 15 % (v/v). The addition of orange juice to the medium greatly stimulated the in vitro growth of explants from several Citrus sp. Tomato juice (30% v/v) has also been used effectively under certain conditions. Other complex organic supplements including banana fruit extract, fish emulsion have been added in the orchid media.

Environmental Factors

Several factors influence the growth of plant tissue cultures such as pH, temperature, light intensity and photoperiods. The pH is an important variable in the culture medium. Plant cells in culture require an acidic pH, and an initial pH of 5.5-5.8 which is optimum. The growth rate of tissue explant is closely relates to the prevailing temperature. Growth may occur at the temperatures below 200o C. A temperature between 26-28o C is considered optimum for achieving maximum growth. Light, necessary for photomorphogenesis and chlorophyll biosynthesis, is provided by Gro-Lux or similar sources or white fluorescent tubes at intensity of 300-10,000 lux. The light is given continuously or photoperiods for 8-16 hours.

1. Plants

In theory, any part obtained from any plant species can be employed to induce callus tissue, however the successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction, as compared to monocotyledons; the callus of woody plants generally grow slowly. Stems, leaves, roots, flowers, seeds and any other parts of plants are used, but younger and fresh explants are preferable as explant materials.

Explants obtained must be sterilized using ethanol, sodium hypochlorite and/or other chemicals to remove all microorganisms from the materials and a typical sterilization procedure will be described later as an example.

Table 5-4 - Media for Plant Tissue and Cell Cultures (mg/L)

Components	Murashige-Skoog (1962)	White (1963	Gamborg (1968)	Nitsch (1951)	Heller (1953)	Schenk - Hildebrandt (1972)	Nitsch - Nitsch (1967)	Kohlenbach - Schmidt (1975)	Knop (1865)
(NH4)2SO4	-	-	134	-	-	-	-	-	-
MgSO4× 7H2O	370	720	500	250	250	400	125	185	250
Na2SO4	-	200	-	-	-	-	-	-	-
KC1	-	65	-	1,500	750	-	-	-	-
CaC12× 2H2O	440	-	150	25	75	200	-	166	-
NaNO3	-	-	-	-	600	-	-	-	-
KNO3	1,900	80	3,000	2,000	-	2,500	125	950	250
Ca(NO3)2× 4H2O	-	300	-	-	-	-	500	-	1,000
NH4NO3	1,650	-	-	-	-	-	-	720	-
NaH2PO4× H2O	-	16.5	150	250	125	-	-	-	-

Components	Murashige-Skoog (1962)	White (1963	Gamborg (1968)	Nitsch (1951)	Heller (1953)	Schenk - Hildebrandt (1972)	Nitsch - Nitsch (1967)	Kohlenbach - Schmidt (1975)	Knop (1865)
NH4H2PO4	-	-	-	-	-	300	-	-	-
KH2PO4	170	-	-	-	_	-	125	68	250
FeSO4× 7H2)	27.8	-	27.8	-	-	15	27.85	27.85	-
Na2EDTA	37.3	-	37.3	-	-	20	37.25	37.25	-
MnSO4× 4H2O	22.3	7	10 (1 H2O)	3	0.1	10	25	25	-
ZnSO4× 7H2O	8.6	3	2	0.5	1	0.1	10	10	-
CuSO4× 5H2O	0.025	-	0.025	0.025	0.03	0.2	0.025	0.025	-
H2SO4	-	-	-	0.5	-	-	-	-	-
Fe2(SO4)3	-	2.5	-	-	-	-	-	-	-
NiC12× 6H2O	-	-	-	-	0.03	-	-	-	-
CoC12× 6H2O	0.025	-	0.025	-	-	0.1	0.025	-	-
A1C13	-	-	-	-	0.03	-	-	-	-
FeC13× 6H2O	-	-	-	-	1	-	-	-	-
FeC6O5H7× 5H2O	-	-	-	10	-	-	-	-	-
K1	0.83	0.75	0.75	0.5	0.01	1.0	-	-	-
H3BO3	6.2	1.5	3	0.5	1	5	10	10	-
Na2M0O4× 2H2O	0.25	-	0.25	0.25	-	0.1	0.25	0.25	-
Sucrose Glucose	30,000 -	20,000 -	20,000 -	50,000 or 36,000	20,000 -	30,000 -	20,000~ 30,000 -	10,000 -	- -
Myo-Inositol	100	-	100	-	-	1,000	100	100	-
Nicotinic Acid	0.5	0.5	1.0	-	-	0.5	5	5	-
Pyridoxine HC1	0.5	0.1	1.0	-	-	0.5	0.5	0.5	-
Thiamine HC1	0.1-1	0.1	10	1	1	5	0.5	0.5	-
Ca-Pantothenate	-	1	-	-	-	-	-	-	-
Biotin	-	-	-	-	-	-	0.05	0.05	-
Glycine	2	3	-	-	-	-	2	2	-
Cysteine HC1	-	1	-	10	-	-	-	-	-
Folic Acid	-	-	-	-	-	-	0.5	0.5	-
Glutamine	-	-	-	-	-	-	-	14.7	-

1. Preparation of Media

To prepare the medium, many researchers mix the stock solutions which were made previously since the medium compositions are generally complicated (30).

For example, MS medium is prepared as follows:

a. MS-Micronutrient stock solution (store in freezer)
Ingredient mg/100 ml
H3BO3 620
MnSO4 4H2O 2230
ZnSO4 7H2O 860
Na2MoO4 2H2O 25
CuSO4 5H2O 2.5
CoCl2 6H2O 2.5

b. Vitamins (store in freezer) Vitamins mg/100 ml
Nicotinic acid 100
Thiamine HCl 1,000
Pyridoxine HCl 100
Myo-Inositol 10,000

c. Calcium chloride
CaCl2 2H2O 15 g/100 ml

d. Potassium iodide (store in amber bottle in refrigerator)
KI 75 mg/100 ml

e. 2,4-D (2.2 mM)
Dissolve 50 mg 2,4-D in 2 to 5 ml ethanol, heat slightly and gradually dilute to 100 ml with water. (Store in refrigerator).

f. NAA (2.8 mM)
Prepare the same as 2,4-D above.

g. Kinetin (1 mM)
Dissolve 21.5 mg of kinetin in a small volume of 0.5 N HCl by heating slightly and gradually diluting to 100 ml with distilled water. (Store in refrigerator) Similar procedures can be used for other cytokinins.

A certain volume of each stock solution is mixed and an appropriate carbon source is added to the mixture. After pH is adjusted to around 5.5 with 0.2 N K0H or 0.2 N HCl, distilled or deionized water is added to the mixture up to the certain volume required. Agar (0.6 to 1.0% wt/vol) is added for a solid medium.

The medium thus prepared is distributed into vessels such as Erlenmyer flasks (for example, 50 ml of the medium in a 300 ml volume Erlenmyer flask) and sterilized by using an autoclave at 120° C for 15 minutes. The sterilization conditions should be varied based on the volume of the medium and the size of the vessel.

2. Callus Induction

Explants are sterilized with 2% sodium hypochlorite solution and/or 70% ethanol solution. The period of time for submerging the plant materials in these solutions depends upon plant species, their parts and age. For example, a piece of stem of tobacco plant (approximately 3 cm in length) is submerged in 70% ethanol solution of 2-3 minutes and then in 1.2% sodium hypochlorite solution for 10 minutes. The explants should be rinsed with sterilized water.

The stem or any other part of plants thus sterilized is cut to approximately 1 cm in length using a sterilized scalpel and each piece is transferred with tweezers to a solid medium in a flask or a petri-dish. The plant material is incubated aseptically at around 25° C on the solid medium for several weeks or more and a callus is produced. The callus is subcultured by transferring a small piece to fresh solid medium. After several subsequent transfers, the callus becomes soft and fragile.

3. Suspension Culture

The growth rate of the suspension cultured cells is generally higher than that of the solid culture. The former is more desirable particularly in production of useful metabolites in a large-scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmyer flask and the vessel placed on a rotary or reciprocal shaker. The culture conditions depend on plant species and other factors, but in general, the cells are cultivated at 100 r.p.m. on a rotary shaker at 25° C; some researchers are fond of much slower or faster speeds. By subculturing for several generations, a fine cell suspension culture containing small cell aggregates and single cells is established. The time required to establish the cell suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The cells in suspension are also used for a large-scale culture with jar-fermentors and tanks.

4. Scaling-up

For commercialization, it is necessary to progress through several stages increasing the volume at each stage until the requisite bioreactor size is attained. In theory, it is anticipated that such large scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation. Generally speaking, the culture period in plant cell cultures is longer than that in microbial cultures, and it is crucial to protect against microbial contamination.

Current Developments in Tissue Culture Area

The benefits of plant tissue culture/ challenges faced in tissue culture field

The benefits of plant tissue culture are extensive in the agricultural world. Micropropagation is favorable to traditional crop breeding methods in many respects, the first being that it allows for the production of huge numbers of plants in a very short period of time. In the Netherlands alone, over 100,000,000 plants are produced using micropropagation each year. Plant tissue culture is also advantageous to growers because the overwhelming number of plants can be produced using the tissue collected from a single parent plant – a plant which itself remains unharmed in the tissue harvesting process. Crop production through micropropagation also eliminates the possibility of any interruption in the growing season because it can be carried out inside the carefully regulated environment of a greenhouse. Because the chemical and physical environment inside a greenhouse can be closely monitored, any lull in production that might typically occur as a result of seasonal change can be avoided.

In theory, it is anticipated that such large scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation.

Nevertheless, there are some significant differences between microbial and plant cell cultures that must be considered when attempting to apply plant cell cultures to the available technology. Table 1 shows a comparison of some of the characteristics of plant and microbial cultures of relevance to fermentation.

This table serves to demonstrate some of the problems that can be encountered with plant cell cultures. The sensitivity to shear is due both to the large size of the cells and to the relatively inflexible cellulose cell wall. Thus, with normal blade impellers the cells may twist which will inhibit mitoses and, for this reason, air-lift fermentors are recommended by some researchers. The large size of the plant cell contributes to its comparatively high doubling time (12 h - several days), which thus prolongs the time required for a successful fermentation run.

The vacuole is the major site of product accumulation, and since product secretion is uncommon, the high metabolite yields seen in microorganisms that secrete product (thereby removing product inhibition of biosynthesis) cannot be expected. There is some ongoing research on membrane

permeabilization of plant cells which may serve to relieve the constraints of product inhibition by facilitation of leakage into the extracellular medium. If this would also permit recycling of the biomass (e.g. via immobilization) it would help reduce production costs (1). The low aeration requirement for plant cells is an advantage over microbial cultures in general. In addition, the high cost of running a fermentation vessel over several weeks should be considered, although media costs are much less than those of animal cell cultures.

Table 5-5 Characteristic of Microbial and Plant Cell Relevant to Fermentation

CHARACTERISTICS	MICROORGANISM	PLANT CELL
Size	2 u	>10 u
Shear stress	Insensitive	Sensitive
Water content	75%	>90%
Duplication time	<1 hour	days
Aeration	1-2 vvm	0.3 vvm
Fermentation time	Days	Weeks
Product accumulation	Medium	Vacuole
Production phase	Uncoupled	Often growth-linked
Mutation	Possible	Requires haploids
Medium cost ($) (MS medium)	8-9/m	65-70/m

In addition to the problems outlined above, concerned with fermentation technology, there are also considerable hurdles to be overcome at the biochemical level. The two major problems concern poor expression of products and instability of cell lines.

Cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant and these quantitative and qualitative features may change with time. The poor product expression is often attributed to a lack of differentiation in cultures (2). On the other hand, there are cases of cultures that over-produce metabolites compared with the whole plant (Table 2).

There are a number of examples of cultured cells producing metabolites not observed in the plant, eg. *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid (3). It has become apparent that the choice of original plant material having high yields of the desired phytochemical may be important in establishing high-yielding cultures (4). Furthermore, the need to repeatedly screen for high-producing lines (due to inherent instability of cell lines) has been emphasized, although the nutritional composition of the medium is also important (2). Thus, a variety of approaches are being investigated by many researchers to increase productivity of useful plant metabolites in plant cell cultures as seen in another section in this review.

Table 5-6 Secondary Metabolites Produced in High Levels by Plant Cell Cultures

COMPOUND	PLANT SPECIES	YIELDS (% DRY WT)		CULTURE TYPE*
		CULTURE	PLANT	
Shikonin	Lithospermum erythrorhizon	20	1.5	s
Ginsenoside	Panax ginseng	27	4.5	c
Anthraquinones	Morinda citrifolia	18	0.3	s
Ajmalicine	Catharanthus roseus	1.0	0.3	s
Rosmarinic acid	Coleus blumeii	15	3	s
Ubiquinone-10	Nicotiana tabacum	0.036	0.003	s
Diosgenin	Dioscorea deltoides	2	2	s
Benzylisoquinoline Alkaloids	Coptis japonica	11	5 - 10	s
Berberine	Thalictrum minor	10	0.01	s
Berberine	Coptis japonica	10	2 - 4	s

COMPOUND	PLANT SPECIES	YIELDS (% DRY WT)		CULTURE TYPE*
		CULTURE	PLANT	
Berberine	Coptis japonica	10	2 - 4	s
Anthraquinones	Galium verum	5.4	1.2	s
Anthraquinones	Galium aparine	3.8	0.2	s
Nicotine	Nicotiana tabacum	3.4	2.0	c
Bisoclaurine	Stephania cepharantha	2.3	0.8	s
Tripdiolide	Tripteryqium wilfordii	0.05	0.001	s
* s = suspension; c = callus				

Micropropagation will be crucial to the agricuture industry in the future because it is used to produce plants which have been genetically modified and selected for their ability to resist certain indigenous environmental stresses. Currently, scientists and members of the agricultural community have joined forces to investigate the possibility of creating lines of tomatoes that possess increased salt tolerance (to be grown in areas in which the soil is high in salinity), plants that are completely resistant to various viral, bacterial, algal, and fungal infections, tobacco plants whose leaves can withstand freezing temperatures, and crops that are entirely resistant to harmful and destructive insects.

The practice of plant tissue culture has changed the way some nurserymen approach plant propagation. In the recent past, the applicability of this technology to the propagation of trees and shrubs has been documented. Some firms have established tissue culture facilities and commercial scale operations are presently in operation for the mass propagation of apples, crabapples, rhododendrons, and a few other selected woody species. The intent of this research update is to briefly examine "what is being done" and to explore "what can be done" with regard to the tissue culture of ornamental plants. Such a consideration necessarily includes an overview of tissue culture as a propagation tool. The major impact of plant tissue culture will not be felt in the area of micropropagation, however, but in the area of controlled manipulations of plants at the cellular level in ways which have not been possible prior to the introduction of tissue culture techniques.

The Art and Science of Micropropagation

Of all the terms which have been applied to the process, "micropropagation" is the term which best conveys the message of the tissue culture technique most widely in use today. The prefix "micro" generally refers to the small size of the tissue taken for propagation, but could equally refer to the size of the plants which are produced as a result.

Micropropagation allows the production of large numbers of plants from small pieces of the stock plant in relatively short periods of time. Depending on the species in question, the original tissue piece may be taken from shoot tip, leaf, lateral bud, stem or root tissue (Fig. 1). In most cases, the original plant is not destroyed in the process — a factor of considerable importance to the owner of a rare or unusual plant. Once the plant is placed in tissue culture, proliferation of lateral buds and adventitious shoots (Fig. 2) or the differentiation of shoots directly from callus (Fig. 3), results in tremendous increases in the number of shoots available for rooting. Rooted "microcuttings" or "plantlets" of many species have been established in production situations and have been successfully grown on either in containers or in field plantings. The two most important lessons learned from these trials are that this methodology is a means of accelerated asexual propagation and that plants produced by these techniques respond similarly to any own-rooted vegetatively propagated plant.
Micropropagation offers several distinct advantages not possible with conventional propagation techniques. A single explant can be multiplied into several thousand plants in less than one year. With most species, the taking of the original tissue explant does not destroy the parent plant. Once established, actively dividing cultures are a continuous source of microcuttings which can result in plant production under greenhouse conditions without seasonal interruption. Using methods of micropropagation, the nurseryman can rapidly introduce selected superior clones of ornamental plants in sufficient quantities to have an impact on the landscape plant market.

Plant Improvement Through Tissue Culture

In introducing this research update, it was mentioned that the major impact of tissue culture technology would not be in the area of micropropagation, but rather in the area of controlled manipulations of plant germplasm at the cellular level. The ability to unorganize, rearrange, and reorganize the constituents of higher plants has been demonstrated with a few model systems to date, but such basic research is already being conducted on ornamental trees and shrubs with the intent of obtaining new and better landscape plants.

Selection of Plants With Enhanced Stress or Pest Resistance

Perhaps the most heavily researched area of tissue culture today is the concept of selecting disease, insect, or stress resistant plants through tissue culture. Just as significant gains in the adaptability of many species have been obtained by selecting and propagating superior individuals, so the search for these superior individuals can be tremendously accelerated using in vitro systems. Such systems can attempt to exploit the natural variability known to occur in plants or variability can be induced by chemical or physical agents known to cause mutations.

All who are familiar with bud sports, variegated foliage and other types of chimeras have an appreciation for the natural variability in the genetic makeup or expression in plants. Chimeras are the altered cellular expressions which are visible, but for each of these which are observed many more differences probably exist but are masked by the overall organization of the plant as a whole. For example, even in frost-tender species, certain cells or groups of cells may be frost hardy. However, because most of the organism is killed by frost, the tolerant cells eventually die because they are unable to support themselves without the remainder of the organized plant. Plant tissues grown in vitro can be released from the organization of the whole plant through callus formation. If these groups of cells are then subjected to a selection agent such as freezing, then those tolerant ones can survive while all those which are susceptible will be killed. This concept can be applied to many types of stress as well as resistance to fungal and bacterial pathogens and various types of phytotoxic chemical agents. The goal of selecting such resistant cell lines would be to reorganize whole plants from them which would retain the selected resistance (Fig. 4). Current research in this area extends across many interests including attempts to select salt tolerant lines of tomato, freezing resistant tobacco plants, herbicide resistant agronomic crops, and various species of plants with enhanced pathogen resistance. Imagine, if you will, the impact of a fireblight-resistant Bartlett pear, a clone of pin oak for alkaline soils, or a selection of southern magnolia hardy to zone 4!

Tissue Culture and Pathogen Free Plants

Another purpose for which plant tissue culture is uniquely suited is in the obtaining, maintaining, and mass propagating of specific pathogen-free plants. The concept behind indexing plants free of pests is closely allied to the concept of using tissue culture as a selection system. Plant tissues known to be free of the pathogen under consideration (viral, bacterial, or fungal) are physically selected as the explant for tissue culture. In most cases, the apical domes of rapidly elongating shoot tips are chosen (Fig. 5). These are allowed to enlarge and proliferate under the sterile conditions of in vitro culture (Fig. 6) with the resulting plantlets tested for presence of the pathogen (a procedure called indexing). Cultures which reveal the presence of the pathogen are destroyed, while those which are indexed free of pathogen are maintained as a stock of pathogen-free material. Procedures similar to these have been used successfully to obtain virus-free plants of a number of species and bacteria-free plants of species known to have certain leaf spot diseases. The impact of obtaining pathogen-free nursery stock can only be speculative, since little research documenting viral, bacterial, or fungal diseases transmitted through propagation of woody ornamentals is available.

Somatic Hybridization

The ability to fuse plant cells from species which may be incompatible as sexual crosses and the ability of plant cells to take up and incorporate foreign genetic codes extend the realm of plant modifications through tissue culture to the limits of the imagination. Most such manipulations are carried out using plant “protoplasts”. Protoplasts are single cells which have been stripped of their cell walls by enzymatic treatment. A single leaf treated under these conditions may yield tens of millions of single cells, each theoretically capable of eventually producing a whole plant. This concept has fueled speculation as diverse as the possibilities of obtaining nitrogen-fixing corn plants on the one extreme to discovering a yellow-flowered African violet on the other extreme.

The observation that has provided the impetus for most of this research is that when cells are stripped of their cell walls and brought into close contact, they tend to fuse with each other (Fig. 7). This “somatic hybridization” is not subject to the same incompatibility problems that limit traditional plant breeding strategies. It is conceivable then that one could hybridize a Juneberry with a crabapple or a plum, but the fundamental research required to demonstrate such an event has yet to be conducted.

The potential use of somatic hybridization to bring about novel combinations of genetic material has been demonstrated in the genera Petunia and Nicotiana. Research funded in part by the Horticultural Research Institute at the University of Wisconsin is investigating the feasibility of using such techniques with woody species. Brent McGown and co-workers have succeeded in obtaining naked cells from tissue cultures of Betula and Rhododendron, but as of yet, they have neither obtained plants from single cells not achieved cellular fusion. However, further research in this area promises to have a tremendous impact on our concepts of woody plant diversity. Just as remarkable as the idea of fusing plant protoplasts is the idea of incorporating foreign genetic material into the genetic code of plant cells. Such transformations have been carried out in the so-called “gene-splicing” experiments where the information for making insulin was incorporated into bacteria. Not only is the desired information transmitted to succeeding generations of bacteria, but the bacterial cultures become synthesizers of insulin as well. Plant cells can be made to take up foreign genetic codes, but evidence that this can be transmitted into the daughter cells and serve the intended function is lacking. What if, for example, the genetic information for accumulating a very high sugar content is incorporated into a clone of sugar maples? One could think of enough what ifÕs in this category to fill several volumes!

Tissue Culture Methods for Virus Eradication

Incubation of plants or of cultures at relatively high temperatures (32o to 40o C) either alone or in combination with the excision and culture of very small stem tips is the only method of ridding infected cultivars of their viruses.

Ideally, we would like to excise and culture the apical meristem of an infected plant because this region is most likely to be free of viruses. In fact, this would involve a very delicate dissection and such small explants are difficult to grow in culture. Instead, we excise a very small stem tip which has, in addition to the apical meristem, one or two leaf primordia. These are much easier to grow in culture and, although a high magnification stereomicroscope is still needed, their dissection is not so difficult.

However, some viruses invade the apical meristem and, in these cases, it is necessary to apply heat treatments to the plant. In the past, this has meant heat treatment of the whole plant followed by stem tip culture. An easier method involves the application of the heat treatment to aseptic cultures. A generalized protocol is as follows:-

1. Disinfest plant material and culture buds;
2. Discard contaminated cultures and rear aseptic cultures until they are small plants;
3. Expose these aseptic plants to elevated temperatures during incubation for various times (to be tested);
4. Excise small stem tips from these incubated plants and incubate in culture at 25o C;
5. Incubate until each plant is quite large and test for presence of viruses;
6. If free of detectable viruses, re-culture from this (or these) plant(s) and multiply to obtain a population of virus-free clonal material.

Note:- Meristem Culture is rarely practised to eradicate viruses and even more rarely in connection with orchids. Orchid propagators using tissue culture techniques nevertheless describe their procedures as either “meristem culture” or “meristemming”, and their products as “mericlones”.

Anther and Pollen Grain Culture

The culture of immature anthers and pollen grains is done so as to induce the pollen grains to develop into multicellular forms, particularly into embryos, with half the normal number of chromosomes for the species. When such haploid embryos are treated with chromosome doubling agents, e.g. colchicine, their normal chromosome number is restored (and thus their fertility) and, in addition, the plants are pure lines. Pure line formation is a natural tendency for self fertilizing species and can be obtained with cross fertilizing species with repeated in-breeding for 10 or more generations.

So far the induction of haploid plant formation from anther and pollen grain cultures has been successful mainly with naturally self fertilizing species and thus, on chromosome doubling, are in theory very similar if not identical with the parents. However, by first crossing many lines from a self fertilizing species, new combinations of genes are formed, and haploid plants produced by anther culture from such crosses could be an extremely valuable and quick way of obtaining pure lines of these new combinations.

If we can find out how to obtain haploid plants from anther culture of cross fertilizing species, they also could be extremely valuable relative to breeding programmes and the selection of improved strains.

Protoplast Culture

Enzymatic removal of the mainly cellulose wall surrounding a plant cell results in a protoplast. When these are cultured, a new wall is synthesized thus restoring the original state of the plant cell. However, during the period that the protoplast is wall-less, there is the possibility:-

1. Of adding foreign genetic material to the protoplast,
2. Of fusing the protoplasts of two cultivars or even species to form hybrid cells.

The latter is called Parasexual Hybridization and could overcome, by avoidance, certain types of incompatibility problems experienced with attempts at sexual hybridization.

Both protoplast culture and the culture of immature anthers and pollen grains are not part of normal micropropagation programmes. Furthermore, since callus and suspension cultures are presently a part of both of these special forms of tissue culture, our knowledge of how to control genome instability and of how to induce either organogenesis or embryogenesis would need to be greatly increased before we would be in a position to exploit the potentialities of these techniques to the full.

Factors Affecting Plant Tissue Culture Success

Plant Material

The influence of plant material on growth and development depends on several factors including genotype, the age of explant, the size of the explant and the method of inoculation. There is a wide range of regenerative capacity in plant kingdom. Dicotyledons generate better than monocotyledons, and gymnosperms have very limited regenerative capacity (except when juvenile). Moreover, there are very great differences in cell division and regenerative capacity between plants within a single species. If a species regenerates organ easily in vivo, then almost the same can be expected in vitro. The age of explant also affects plant tissue culture success. Young and soft tissues are generally more amenable for culture than older woody tissues. Although the isolated part of the plant has its own supply of food reservation and hormones but it is obvious that the larger the plant part, the easier inducing growth and regeneration. Very small structures such as cells, clumps of cells, and meristem are much more difficult to induce growth than larger structures such as leaf, stem or tuber explants. Explants can be inoculated on the culture media in different way: polar (straight up, with the physiological base in the medium) or apolar (upside down, physiological base out of the medium and top in the medium). The method of inoculation is especially important; with apolar inoculation roots and shoots generally regenerate more easily and more rapidly.

Totipotency

Introduction

TOTIPOTENCY - is the ability for undifferentiated plant cells to develop into whole plants or plant organs in vitro when given the correct conditions.

NB. not all plant cells are totipotent. However, there are a sufficient number of totipotent cells in the plant (e.g. in the pith) for this type of development to take place.

Cells at a relatively early stage of development e.g. non-dividing undifferentiated parenchyma meritematic and embryonic tissues that are undetermined. i.e. they are capable of switching to different pathways of development depending upon the environments imposed upon them. Such undetermined cells can also rapidly proliferate to produce cell masses known as callus.

Callus is often naturally produced by plant tissue in response to wounding.

Thus, totipotent cells exhibit a high degree of plasticity in their response to physical and environmental stimuli. This is a survivial technique for the dangers that vascular plants face, such as grazing, attack by pathogens and the requirement to proliferate by vegetative propagation.

If we use the correct culture medium we can induce growth of callus in plant tissue explants (sections of plant tissue from:- buds, roots, stems, leaves, seeds, anthers etc).

For example, tissue explants can be induced to form callus by the application of plant growth regulators (or plant "hormones"). An Auxin to Cytokinin concentration of 3.0 to 0.2 mg l-1 can induce plant cells to form callus. Callus can be inuced to form roots in an auxin to cytokinin concentration of 3.00 to 0.02 mg l-1, and to induce shoot formation in 0.03 to 1.00 mg l-1. You will perform this type of experiment over the next few weeks.

Such techniques can also be used to induce callus-type growth in single cells or protoplasts transformed of novel genotype. This callus can then be induced to form roots and shoots, transplanted into soil and thus developed into a plant with a novel genotype.

More significantly, some callus cultures, under certain nutritional and hormonal conditions can be induced to develop bipolar embryoids. This is known as somatic embryogenesis and follows a sequence through pro-embryoid, globular and torpedo stages. The synthetic auxin 2,4-D is used to form calluses from tissue explants. The callus is then transferred to a 2,4-D free medium supplimented with reduced froms of nitrogen (in the form of NH4+ salts or amino acids).

Such manipulations in plant tissue culture have been particularly sucessful in the case of Tobacco and Carrot tissue. However, not all crop tissues can be manipulated as readily as these species and much intensive research has been employed in optimising plant tissue culture conditions in agricultural crops. This is particularly true of the cerial crops (Monocotyledenous plants) which are particulaly difficult to manipulate in culture.

Suspension Cultures

If callus is treated with pectinase (breaks down the middle lanemella of the plant cell wall and separates plant cells) then suspension cultures of single cells can be theoretically formed.

It was intially thought that if plant cells were grown in suspensions of individual cells, then some of the techniques and growth kinetics applied to microbial suspension cultures could be applied. However, this is not the case as plant cells tend to naturally clump together and they grow toether in tha natual environment. Therefore, plant suspension cultures are not totally sucessful.

However, plant suspension cultures of single cells or microcalli are useful as a source of cells for protoplast isolation. Others, such as the Tobacco bright yellow 2 cell lise are useful a model system to investigate the effect of different treatments on cell division and cell mortality.

Plant Cell Protoplast Culture

The isolation procedure and an introduction to plant cell protoplasts can be found in Dodds and Robert.

Protoplasts are plant cells without cell walls or "naked plant cells". These can be manipulated either by inserion of foreign DNA by some mechanism or by protoplast fusion to give novel genotypes. After a few days (in some cases hours) in culture protoplasts form new cell walls. Following this stage protoplasts can be regenerated into microcalli, calli, and ultimately whole plants.

Protoplasts have also been used to study the regeneration of the cell wall.

Shoot Meristem Culture for the Production of Virus-free Plants.

General aseptic techniques are used in conjunction with meristem tip culture to produce virus-free crops. [for details, Ingram D S & Helegeson J P (1980) eds. Tissue Culture Methods for Plant Pathologists. Blackwell Scientific Publications, Oxford]. Reductions in nematode, bactrial infestations can be achieved by culturing pathogen-free parts of the plant. However, since the majority of plant viruses infect plants in a systemic manner, this type of approach is not sufficient to produce virus-free plants. To obtain virus-free plants cultures can be obtined in two ways:-

1. cultures of small growing shoot tips, the apical meristems consisting of less than 500 um of the apical dome and one to three leaf primordia.
2. cultures of larger shoot tips excised from donor plants or dormant propagules that have been exposed to hot-air treatments of 50-60 C for 5-10 minutes to reduce the level of viral repliation in the explants.
3. cultures of 1 or 2 grown on media containing anti-viral compounds such as malachite green or thiouracil.

Plant viral infections are thansmitted thoruoghout the plant via the vascular system. However, the shoot meristem is contunually renewing tissue, which is virus-free when formed. Thus, only tissue near the vascular elements becomes infected and isolated shoot meristems are virus-free.

Meristem tips are carefully dissected away from the apical or lateral shoot buds under a dissection microscope. If dissection is carried out under aseptic conditions, most meristems can be placed on the media without sutface sterilisation. Meristem tips can be cultured on either liqid or semi-solid media. The media is generally based on White's media. After between 50 - 100 days the few plantlets or adventitous shoots produced can be further micropropagated.

The technique has been used for:- Rhubarb crops, Chrysanthemum, narcissus, carnations and other flower crops, Pelargonum, potato, and strawberries.
In the case of potato, virus free "seed tubers" are commercially produced and marketed to the farmers.

The Use of Plant Tissue Culture in Crop Breeding

Some in vitro techniques can be used for crosses between plants of different species or sub-species that would not normally cross in natrual conditions. Additionally tissue culture methods can be used to rescue embryos that would not have developed normally from ovules due to various incompatability mechanisms. Furthermore, tissue culture offeres the chance to use valuable types of breeding material e.g. haploid cell cultures, to be regenerated in a relatively short time. Some breeding programmes such as those for rice, barley and tobacco regularly utilise anther and/or pollen culture techniques. Potential also exists for single gene transfer using recombinant DNA techniques.

Somatic Hybridisation

Isolated protoplasts can be induced to fuse. Non-specific fusion of protoplasts from the same or different species can be induced using two methods:-

1. Use of Chemical Fusing Agents. such as Polyethylene glycol (PEG) in the presence of high Ca++ and a high pH. However, this may have an effect on protoplast viability.
2. By electical depolarisation (electro fusion). surface charges of plasmalemma membranes of protoplasts are altered with short-pulses of direct current. There is little effect on protoplast viability. Gives an efficient high frequency of fusion.

protoplast fusion can be used to:-

1. Produce fertile diploid somatic hybrids of sexually incompatable species.
2. Produce heterozygous lines within a single species which could normally only be propagated by vegetative means e.g. potato and other root crops.
3. Transfer of limeted parts of the genome from one species to another by the formation of heterkaryons (see ohp)
4. production of novel interspecific and intergenetic crosses between plants impossible to hybridise conventionally. e.g. pp of potato and tomato have been fused to give a "pomato" [Melchers et al (1978) Carlsberg Research Communications 43 203-218]. However, these types of combinations are rarely fertile and seldom produce viable seed.

Production of hebicide resistance using protoplasts.

Resistance to the herbicide atrazine has been transferred from bird's rape (*Brassica campestris*) to oilseed rape (*Brassica napus*) via protplast fusion [Beversdorf *et al.* (1980) Canadian Journal of Genetics and Cytology 22 167-172].

Production of Mutants Using Plant Tissue Culture.

Mutagens can be applied to plant cell cultures (suspension or protoplast culture). generated mutants can be tested for tolerance to salt or fungal toxins. If cultures grow with this selcetion pressure then the plants that they produce are ofter resistant to the same thing. This system has been used to generate salt-tolerant tobacco plants and to develop corn resistant to the *Helmintosporium* fungus.

Somaclonal Variation

There is a type of natural mutagenesis that exists within plant tissue culture. Somaclonal variation covers types of variations that occcur in plants regerated from cultured cells or tissues. e.g. sugar cane calli were screened and selected for resistance to fungal disease. The resistant somaclones retained their resistance to the fungal pathogen. Increases in the yield of sugar-cane have also been obtained from the screening of calli. Not only did the tissue culture cloning improve sugar cane yields but it is also used to produce novel types of sugar cane useful for animal feedstocks or as substrates for alcohol fermentation.

More importantly, resistance to early blight (caused by *Alternaria solani*) was developed from protoplasts of the potato variety Russet Burbank. This line also showed greater yield and tuber uniformity.

Among the mechanisms that are responsible for somaclonal variation are gross karyotypic changes that accompany callus formation, including chromasomal re-arrangements, somatic crossing over, sister chromatid exchange, transposable elements and gene amplification.

Summary

Plant tissue culture research is multi-dimensional. While most nurserymen have been introduced to the techniques and advantages of micropropagation, few have ventured to use it as a propagation tool. The applicability of micropropagation for woody trees has been demonstrated as feasible since all aspects of the technology have confirmed the fact that trees produced by this method look like and grow like their counterparts produced by traditional methods of cloning.

Other dimensions of tissue culture research have been less well publicized. The potential for selecting pathogen free plants, for selecting stress-tolerant and pathogen-resistant clones of plants, and the novel genetic combinations to be achieved through somatic hybridization are all lines of research which can have a profound impact on the nursery industry.

Notes